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[12] Description of Invention Patent Application

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Title of Invention: Super-insulinotropic Peptides and their Applications

Abstract

This invention provides a type of super-insulinotropic peptides, characterised in that: the amino acid sequence is NH_2-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-COOH, in which AA1 represents neutral or hydrophobic amino acid; AA2 represents acidic amino acid with side chain negatively charged; AA3 represents hydrophobic amino acid; AA4 represents basic amino acid with side chain positively charged; and AA5 represents amino acid with side chain carrying benzene ring. Super-insulinotropic peptides introduced by this invention have higher insulinotropic activity and stability than prior art Exendin-4 (EX-4), and much higher stability than prior art glucagon-like peptide-1 (GLP-1). Super-insulinotropic

peptides introduced by this invention can enhance drug efficacy while reducing required dosage thereby cutting toxic side effects, and can therefore be used in potential clinical applications in the treatment of diabetes mellitus and obesity.

GLP-1: HAEGTFTSDVSSYLEGQAAKEFIAWLVKG

EX-4: HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS

SIP-1: HXEGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS

SIP-2: HGEGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS

SIP-3: HSQGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS

SIP-4: Ac-HXEGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS

Claims

1. A type of super-insulinotropic peptides, characterised in that: the amino acid sequence is NH2-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-COOH, in which AA1 represents neutral or hydrophobic amino acid; AA2 represents acidic amino acid with side chain negatively charged; AA3 represents hydrophobic amino acid; AA4 represents basic amino acid with side chain positively charged; and AA5 represents amino acid with side chain carrying benzene ring.
2. Super-insulinotropic peptides as described in Claim 1, characterised in that: aforesaid AA1 may be D-Ala, Ser, β-Ala or Gly; aforesaid AA2 may be Glu, Gln or Asn; aforesaid AA3 may be Val, Leu or Ile; aforesaid AA4 may be Lys or Arg; and aforesaid AA5 may be Tyr or Phe.
3. Super-insulinotropic peptides as described in Claim 2, characterised in that: aforesaid AA1 may be D-Ala or Gly; aforesaid AA2 may be Glu or Gln; aforesaid AA3 is Val; aforesaid AA4 is Lys; and aforesaid AA5 is Tyr.
4. Super-insulinotropic peptides as described in Claim 3, characterised in that: aforesaid AA1 is D-Ala; and aforesaid AA2 is Glu.
5. Super-insulinotropic peptides as described in Claim 1, characterised in that: the C-terminal may have amino group, hydrophobic group or macromolecule vehicle group as protective group.
6. Super-insulinotropic peptides as described in Claim 5, characterised in that: the C-terminal has amino group as protective group.
7. Super-insulinotropic peptides as described in Claim 1, characterised in that: the N-terminal may have acetyl group, hydrophobic group or macromolecule vehicle group as protective group.
8. Super-insulinotropic peptides as described in Claim 7, characterised in that: the N-terminal has acetyl group as protective group.
9. Super-insulinotropic peptides as described in Claim 5 or Claim 7, characterised in that: aforesaid hydrophobic group may be benzyloxycarbonyl, dansyl or tertiary butyloxycarbonyl; aforesaid macromolecule vehicle group may be lipid-fatty acid conjugate, polyethylene glycol or carbohydrate.

10. Super-insulinotropic peptides as described in Claim 1 – 6, characterised in that: the amino acid sequence is NH_2-His D-Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.
11. Super-insulinotropic peptides as described in Claims 1 – 6, characterised in that: the amino acid sequence is NH_2-His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.
12. Super-insulinotropic peptides as described in Claims 1 – 6, characterised in that: the amino acid sequence is NH_2-His Ser Gln Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.
13. Super-insulinotropic peptides as described in Claims 1 – 6, characterised in that: the amino acid sequence is Ac-His D-Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.
14. A drug compound, characterised in that: it contains one of the super-insulinotropic peptides as described in Claims 1 – 13 and other normal medicinal adjuvant.
15. A drug preparation, characterised in that: it contains one of the super-insulinotropic peptides as described in Claims 1 – 13 or drug compound as described in Claim 14; the form of medication may be injectable powder, injectable preparation, inhaler or oral application.
16. Any of the super-insulinotropic peptides as described in Claims 1 – 13 or drug compound as described in Claim 14 to be used as a drug preparation in the treatment of diabetes mellitus and obesity.

Description

Super-insulinotropic Peptides and their Applications

Technical field

This invention relates to the field of medicine. More specifically, it relates to a type of super-insulinotropic peptides for the treatment of diabetes mellitus, their preparation methods, and drug compounds containing aforesaid super-insulinotropic peptides and their applications.

Background art

Glucagon-like peptide-1 (GLP-1, for which the amino acid sequence is: HAEGTFTSDVSSYLEGQAAKEFIAWLVKG) is an endogenous insulinotropic peptide that is secreted *in vivo* in response to a high plasma glucose concentration. Due to this feature, GLP-1 was looked upon by many as a basis from which to develop a new generation of drugs for the treatment of diabetes. However, results from numerous studies have shown that GLP-1 is not clinically useful as a drug because it is highly unstable and prone to rapid degradation *in vivo*. Studies suggest that the reasons for the extreme unstableness of GLP-1 may be two-fold: (1) the N-terminal His-Ala of GLP-1 is very susceptible to degradation by dipeptidyl peptidase *in vivo* (Deacon et al., Journal of Clinical Endocrinology & Metabolism, 1995, 80:952-57), and (2) the C-terminal structure of the peptide is relatively loose, causing extreme unstableness in peptide molecule structure (Neidigh et al., Biochemistry, 2001, 40(44):13188-200).

By comparing the sequence of GLP-1 with other peptides, it was found that Exendin-4 (EX-4, for which the amino acid sequence is: HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS), a peptide secreted from the saliva of the lizard *Heloderma suspectum*, had a measure of sequence homology with GLP-1. Further studies have shown that it has the same function of stimulating insulin secretion as GLP-1, but is substantially more stable than GLP-1. Due to these advantages, EX-4 has been extensively studied by scientists in many

countries. At present, phase III clinical trial for EX-4 is being carried out in USA primarily in the treatment of Type II (insulin-independent) diabetes.

Results from clinical studies so far have shown that EX-4 has a marked glucose lowering effect, with a very good therapeutic effect for Type II diabetes. Compared with existing antidiabetic drugs such as sulfonylureas, metformin, or insulin, EX-4 has many obvious advantages. Both sulfonylureas and metformin, currently in extensive clinical use, work primarily by stimulating insulin secretion from pancreatic islet β-cells rather than by improving the functioning of pancreatic islet β-cells. With the progression of pathogenetic condition, the pancreatic islet β-cells will gradually lose its ability to secret insulin, diminishing efficacy of aforesaid drugs. This inevitably leads to the use of insulin injection. It is estimated that 10 per cent of diabetic patients are forced to switch to insulin annually. To maintain a normal plasma glucose concentration, it is necessary for those patients to undergo insulin injection everyday and to continually increase dosage. After using insulin for a certain period of time, the activity of insulin receptors *in vivo* may gradually diminish, leading to inefficacy of insulin use. EX-4 can not only stimulate insulin secretion, but also stimulate proliferation and differentiation of pancreatic islet β-cells, and improve the functioning of these cells, thereby fulfilling the purpose of treating diabetes (Movassat et al., Journal of Clinical Endocrinology & Metabolism, 2002, 87(10):4775-81). In addition to its effect on pancreatic islet β-cells, EX-4 has a direct effect on liver cells and muscle cells as well, lowering glucose by inhibiting glucogen degradation and promoting glucogen synthesis through a pathway independent of insulin (Alcantara et al., Archives of Biochemistry & Biophysics, 1997, 341(1):1-7). Also, as exogenous insulin injection lowers glucose at any plasma glucose concentrations, hypoglycaemia may occur when dosage is not appropriate, which may lead to complications such as shock or coma. However, EX-4 does not have aforesaid side effects like insulin, because it only lowers glucose at high plasma glucose concentrations, and exerts no glucose lowering effect at normal plasma glucose concentrations (Vella et al. Diabetologia, 2002, 45(10):1410-5). Furthermore, EX-4 can also suppress appetite by slowing down gastric emptying, which is helpful for the treatment of diabetes (Edwards et al., American Journal of

Physiological Endocrinology & Metabolism, 2001, 281(1):E155-61). This advantage of EX-4 is again absent in other antidiabetic drugs.

However, EX-4 also has its disadvantages, primarily due to its relatively strong side effects, including nausea, vomiting, and even shock. For this reason, its clinical dosage range is kept extremely narrow, with very small dosage. A patient requires two injections daily, which is rather inconvenient. Although the mechanism by which EX-4 exerts its side effects has not been well understood so far, a plausible explanation would be that EX-4, a peptide secreted from the saliva of the lizard *Heloderma suspectum*, is an exogenous substance for human beings.

Therefore, it is very much hoped to develop a new antidiabetic drug with higher activity, better stability and less side effects.

Invention

The purpose of this invention is to provide a type of super-insulinotropic peptides with higher activity, better stability and less side effects, and to optimise them for a new generation of antidiabetic drugs.

Given that GLP-1 has better biological activity and EX-4 has better stability, the inventors have developed a type of super-insulinotropic peptides (SIP), among which four peptides are the most representative embodiments:

SIP-1: HXEGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS
SIP-2: HGEGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS
SIP-3: HSQGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS
SIP-4: Ac-HXEGTFTSDVSKYMEEEAVRLFIEWLKNGGPSSGAPPPS

The inventors have conducted extensive experiments and studies in this direction, with results showing that said type of super-insulinotropic peptides have a much higher stability than GLP-1 as well as a significantly higher biological activity than EX-4. It can therefore be concluded that the super-insulinotropic peptides introduced by this invention can fulfil the purpose of same. Aforesaid research results have facilitated the completion of this invention.

In other words, the technical scheme of this invention is as follows:

A type of super-insulinotropic peptides, characterised in that: the amino acid sequence is NH_2-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-COOH, in which His represents histidine; Ala represents alanine; Glu represents glutamic acid; Gly represents glycine; Thr represents threonine; Phe represents phenyalanine; Ser represents serine; Asp represents aspartic acid; Val represents valine; Tyr represents tyrosine; Leu represents leucine; Gln represents glutamine; Lys represents lysine; Ile represents isoleucine; Trp represents tryptophan; Met represents methionine; Arg represents arginine; Asn represents asparagine; Pro represents proline; AA1 represents neutral or hydrophobic amino acid, it can be D-Ala, Ser, β-Ala or Gly, preferably D-Ala or Gly, with D-Ala as the most preferable; AA2 represents acidic amino acid with side chain negatively charged, it can be Glu, Gln or Asn, preferably Glu or Gln, with Glu as the most preferable; AA3 can be Val, Leu or Ile, preferably Val; AA4 can be Lys or Arg, preferably Lys; and AA5 can be Tyr or Phe, preferably Tyr.

Aforesaid super-insulinotropic peptides, characterised in that: the C-terminal may have amino group, hydrophobic group or macromolecule vehicle group as protective group, with amino group as the preferred option.

Aforesaid super-insulinotropic peptides, characterised in that: the N-terminal may have acetyl group, hydrophobic group or macromolecule vehicle group as protective group, with acetyl group as the preferred option.

Aforesaid super-insulinotropic peptides, characterised in that: aforesaid hydrophobic group may be benzyloxycarbonyl, dansyl or tertiary butyloxycarbonyl; aforesaid macromolecule vehicle group may be lipid-fatty acid conjugate, polyethylene glycol or carbohydrate.

Aforesaid super-insulinotropic peptides, characterised in that: the amino acid sequence may be any of the following:

SIP-1: NH_2-His D-Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

SIP-2: NH_2-His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

SIP-3: NH_2-His Ser Gln Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

SIP-4: Ac-His D-Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

A drug compound, characterised in that: it contains one of the aforesaid super-insulinotropic peptides and other normal medicinal adjuvant.

A drug preparation, characterised in that: it contains one of the aforesaid super-insulinotropic peptides or aforesaid drug compound; the form of medication may be injectable powder, injectable preparation, inhaler or oral application.

This invention also describes how any of the aforesaid super-insulinotropic peptides or drug compound can be used as a drug preparation in the treatment of diabetes mellitus or obesity.

Furthermore, this invention provides a method for preparing super-insulinotropic peptides, characterised in that: the method is solid-phase polypeptide chemical synthesis.

Some issues in the technical scheme of this invention are explained below.

In the amino acid sequence described in aforesaid technique scheme, both NH_2- on the left terminal and -COOH on the right terminal are part of the internal structure of corresponding amino acids (His and Ser, respectively). Thus, they can be omitted in writing, in which case it still means that the left terminal is NH_2- and the right terminal is -COOH. The order of the two groups cannot be reversed.

The -$CONH_2$, on the right terminal of aforesaid amino acid sequences for SIP-1 – SIP-4, is the protective group of the carboxyl terminal. The carboxyl terminal with a protective amino group is so called because -$CONH_2$, a product of the reaction between the internal -COOH and the exotic amino group, performs a protective function on the right terminal (i.e. carboxyl terminal). This is the case for all aforesaid amino acid sequences for SIP-1 – SIP-4. The amino terminal with a protective acetyl group is so called because acetamido- (Ac-), a product of reaction between the acetyl group and the amino group, performs a protective function on the left terminal (i.e. amino terminal). This is the case for aforesaid amino acid sequence for SIP-4.

Below is a detailed description of the invention.

Super-insulinotropic peptides are a heterozygote of GLP-1 and EX-4, with an N-terminal segment same as, or similar to, the N-terminal segment of GLP-1 and a C-terminal segment same as, or similar to, the C-terminal segment of EX-4. The N-terminal segment of GLP-1 is preserved in super-insulinotropic peptides as it is an important segment for receptor binding and performing biological functions. Nevertheless, to protect the peptides against degradation by dipeptidyl peptidase, L-Ala at position 2 needs to be altered. To preserve the insulinotropic activity as much as possible, the L-Ala is replaced by amino acids similar to it, such as those neutral amino acids with short side chains, including D-Ala, Gly and Ser.

Glu at position 3 in GLP-1, which constitutes a part of the recognition site of depeptidase, is changed to amino acids similar to Glu, such as Gln or Asp, thereby enhancing the stability of the peptides without compromising activity.

The amino acids at position 10 are Val in GLP-1 and Leu in EX-4. Both are hydrophobic amino acids with large side chains and have important effects on the biological activity of the super-insulinotropic peptides. Therefore, amino acid at this position is designed as some hydrophobic amino acid with large side chain, such as Val, Leu or Ile.

Structure analysis of EX-4 shows that Lys at position 12 is involved in the formation of ionic linkage, which is conducive to stability. Therefore, amino acid at this position is designed as some basic amino acid with side chain positively charged, such as Lys or Arg.

The amino acids at position 13 in GLP-1 and EX-4 are dramatically different. To preserve the high activity of GLP-1, amino acid at this position in super-insulinotropic

peptides is designed as Tyr, which is same to that in GLP-1, or similar amino acid carrying benzene ring, such as Phe.

Structure analysis shows that the C-terminal in GLP-1 is very loose, and in contrast, the C-terminal in EX-4 is very tight, which significantly enhances the stability of the peptides. Therefore, the C-terminal segment (including amino acids from position 14 to position 39) of super-insulinotropic peptides in this invention is designed as the same as that in EX-4.

To further enhance the stability of the super-insulinotropic peptides, the N-terminal is protected by acetamido-, a product of reaction between the internal amino group and the exotic acetyl group, and the C-terminal is protected by -$CONH_2$, a product of reaction between the internal carboxyl group and the exotic amino group.

This invention provides a method for preparing super-insulinotropic peptides, characterised in that: the method is solid-phase polypeptide chemical synthesis.

In light of their composition features, super-insulinotropic peptides in this invention are prepared by Fmoc solid-phase polypeptide synthesis, and the crude product is then purified using high performance liquid chromatography (HPLC).

The basic principle for solid-phase polypeptide synthesis can be summarised as follows: synthesis is carried out in the direction of C to N and start with the C-terminal residue of the desired peptide, with stepwise addition of amino acids with protected N-terminal and side chain to the growing oligopeptide chain that is attached to insoluble resin. Each cycle, defined as a complete addition of an amino acid to the growing

oligopeptide chain, consists of three steps: (1) remove the protective N-terminal group of preceding amino acid; (2) link C-terminal of posticus amino acid with protective amino group to N-terminal of preceding amino acid by formation of new peptide bond; and (3) filtrate and wash the excess reagents and by-products in the interval between step 1 and step 2. Once the synthesis is completed, the peptide is eluted from the resin and the crude product is obtained.

Solid-phase polypeptide synthesis, by attaching a growing oligopeptide to insoluble resin, has obvious advantages: separation of excess reagents and by-products in the reaction process can be achieved simply by filtration and washing, thereby simplifying the isolation and purification procedures for intermediate products and reducing product losses in the process. These are advantages that cannot be matched by liquid-phase synthesis process. In addition, the operation of filtration and washing is simple, and the entire synthesis process for peptides can be programmed and automated, enormously enhancing production efficiency. Moreover, the super-insulinotropic peptides, with about 39 residues, are long peptides, far exceeding the scope of normal liquid-phase synthesis methods. Therefore, solid-phase polypeptide synthesis is the only option for synthesis of super-insulinotropic peptides.

This invention provides a method for assaying the biological activity of super-insulinotropic peptides.

GLP-1 or EX-4 has a specific stimulatory effect on promoter of insulin gene in rat insuloma RIN cells. Therefore, the biological activity of the peptides can be determined by measuring the amount of insulin secreted into the medium of RIN cells. However, measurement of insulin content in this way requires a specialist assay kit, which is both expensive and complicated to operate. To establish a convenient, reliable and sensitive method for assaying the biological activity of super-insulinotropic peptides, a report gene luciferase is cloned into the downstream sequence of rat insulin gene promoter.

When the cells are stimulated by the peptides, the insulin gene promoter is activated, causing luciferase expression. Thus, the biological activity of the peptides can be determined by measuring luciferase activity in cell lysates.

This invention provides a method for assaying the stability of super-insulinotropic peptides.

To study the stability of super-insulinotropic peptides *in vivo*, the peptides are incubated with human blood plasma at 37□, mimicking *in vivo* conditions. Then add samples into aforesaid cell medium for assaying biological activity. Preserved biological activity of the peptides can be determined by analyzing the increase of luciferase activity in stimulated cell lysates.

This invention provides a drug compound containing super-insulinotropic peptides.

To study the feasibility of using super-insulinotropic peptides as a drug, a drug compound is prepared by adding manicol and human albumin into a solution that contains the peptides, and then using phosphate buffer to adjust the power of hydrogen to neutrality. It is found that, after freezing and drying of aforesaid drug compound, its storage life is significantly extended without compromising its biological activity.

Compared with the prior art in this field, super-insulinotropic peptides introduced by this invention have several benefits: these super-insulinotropic peptides have higher insulinotropic activity and stability than EX-4, and much higher stability than GLP-1. Super-insulinotropic peptides introduced by this invention can therefore enhance drug

efficacy while reducing required dosage, enhancing therapeutic effect while reducing toxic side effects in the treatment of diabetes mellitus and in diet control.

Description of the drawings:

Fig.1 An amino sequence comparison of SIP-1 – SIP-4 with EX-4 and GLP-1;

Fig.2 A purity analysis of super-insulinotropic peptide-1 by high performance liquid chromatography;

Fig.3 A construction diagram of report vector pGL3-RIP-Luc;

Fig.4 A biological activity comparison of SIP-1 – SIP-4 with EX-4 and GLP-1;

Fig.5 A stability comparison of SIP-1 – SIP-4 with EX-4 and GLP-1;

In Fig. 1, SIP-1, 2, 3 and 4 represent super-insulinotropic peptide-1, 2, 3 and 4, respectively, and EX-4 represents Exendin-4. Code designation for amino acids in Fig.1 is as follows. H: His; A: Ala; E: Glu; G: Gly; T: Thr; F: Phe; S: Ser; D: Asp; V: Val; Y: Tyr; L: Leu; Q: Gln; K: Lys; I: Ile; W: Trp; X: D-Ala; M: Met; R: Arg; N: Asn; P: Pro; and Ac: acetyl.

Fig.2 demonstrates a purity analysis of super-insulinotropic peptide-1 by HPLC method after purification procedures. The method for said purity analysis is explained in preferred embodiment 1.

In Fig.3, the target DNA fragment, a rat insulin promoter (RIP) sequence (nucleotides -410 – 9) is amplified from rat genomic DNA by polymerase chain reaction (PCR).

Recognition sites of restriction enzyme BglII and StuI are introduced into both terminus of the target DNA fragment by amplification using oligonucleotide primers. After cleaving with those two restriction enzymes, the DNA fragment is cloned into the upstream sequence of report gene luciferase (Luc). The arrow in final report vector pGL3-RIP-Luc denotes the start site of transcription.

In Fig.4, SIP-1, 2, 3 and 4 represent super-insulinotropic peptides-1, 2, 3 and 4, respectively, and EX-4 represents Exendin-4. The report cell RIN-RIP-Luc is stimulated by super-insulinotropic peptides (10nM) and then lysed to measure luciferase activity. The increased fold of luciferase biological activity is calculated by dividing luciferase activity in cells with stimulation by luciferase activity in cells without stimulation. The greater the result, the higher activity the peptides have. The values shown in Fig.4 are the average from three experiments.

In Fig.5, SIP-1, 2, 3 and 4 represent super-insulinotropic peptides-1, 2, 3 and 4, respectively, and EX-4 represents Exendin-4. After warm incubation with human blood plasma for 2 hours, the peptides are added into a cell medium with report cell RIN-RIP-Luc to measure biological activity. The increased fold of luciferase biological activity is calculated by dividing luciferase activity in cells with stimulation by luciferase activity in cells without stimulation. The values shown in Fig.5 are the average from three experiments.

A more detailed description of the invention is provided below by introducing several preferred embodiments of this invention.

Preferred embodiment 1:

1) Amino acid sequence of super-insulinotropic peptides-1 (SIP-1):

SIP-1: NH_2-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

In this sequence, AA1 is D-Ala, AA2 is Glu, AA3 is Val, AA4 is Lys, and AA5 is Tyr.

2) Preparation of super-insulinotropic peptides-1 using solid-phase polypeptide synthesis method.

Protocol is as follows:

1. Place 1.0g of resin (Rink Amide) in the reactor of the ACT90 solid-phase synthesizer. Wash the resin twice with 10 ml of dichlormethane, methanol and dimethylformamide, respectively. Remove solvent by sucking filtration.

2. Based on 0.8mmol/g of substituent constant (i.e. the mole number of peptide molecule that can be linked to every gram of resin) of resin, dissolve Fmoc-protected first amino acid in the C-terminal of peptide (Fmoc-Ser(tBu)-OH), linker HBTU and diisopropyl acetamide in 10 ml of dimethylformamide in a ratio of 1:3:3:7. Add the solution into the reactor of the solid-phase synthesizer, and agitate for 2 hours at room temperature, then remove the reaction liquid by sucking filtration. Wash the resin twice with dimethylformamide, methanol and dichlormethane respectively, and then remove solvent by sucking filtration.

3. Add 10 ml of piperidine/dimethylformamide (20%, v/v), and agitate for 30 minutes at room temperature to deprotect Fmoc- in the N-terminal, then remove the solvent by sucking filtration. Wash the resin twice with dimethylformamide, methanol and dichlormethane respectively, and then remove the solvent by sucking filtration.

4. Repeat Step 2 and Step 3 above, with the exception of alteration to the Fmoc-protected amino acid in Step 3 in accordance with amino acid sequence in the C to N direction. Continue this cycle until the peptide assembly on resin is complete. Wash resin and dry it in vacuo overnight.

5. Transfer resin to a 250-ml round-bottomed flask. Add trifluoroacetic acid-H_2O (95%:5%) in a ratio of 20ml of trifluoroacetic acid-H_2O per 1g of resin with peptides, and agitate for 1 – 3 hours at room temperature. Separate filtrate from resin by sucking filtration. Add 2000 ml of aether (0□) into filtrate, and centrifuge to separate the precipitation from aether. Dry the precipitation, and thus obtain a crude product of super-insulinotropic peptides-1.

Purification process of the crude product of super-insulinotropic peptides by HPLC method:

Dissolve dry crude product of super-insulinotropic peptides in 1% acetic acid with a final concentration of 20mg/ml. Using reverse phase high performance liquid chromatography method, the crude product of peptides is purified by a series of procedures, including gradient elution for isolation, collection of main peak parts, combining the product, freezing and drying, thus obtaining pure super-insulinotropic peptides-1. Chromatographic conditions are as follows:

Chromatography: Varian liquid phase chromatography ProStarSD-10 and its operation and analysis software;

Chromatographic column: Dynamax Analytical C_{18} chromatographic column (4.6×250mm);

Mobile phase: A: 0.1% trifluoroacetic acid/10% acetonitrile/H_2O; B: 0.1% trifluoroacetic acid/90% acetonitrile/H_2O;

Elution gradient: 30-50% mobile phase B, 30 minutes;

Flow rate: 1.0 ml/min;

Ultraviolet detection wavelength: 220 nm.

After purification of crude product using HPLC, the purity of the peptide is greater than 95% (analysis results are shown in Fig.2).

To verify the correctness of the synthesised and purified product, the purified super-insulinotropic peptide-1 was subjected to mass chromatographic analysis, and the results are as follows:

Molecular formula: $C_{188}H_{283}N_{49}O_{60}S_1$; Theory molecular mass: 4221.71; Measured value: 4222.0.

These results confirm that the synthetic super-insulinotropic peptide-1 is the desired peptide.

3) Biological activity assay for super-insulinotropic peptide-1.

Firstly, to construct a report vector pGL3-RIP-Luc by cloning report gene luciferase into the downstream sequence of rat insulin gene promoter (see Fig.3 for plasmid construction). Protocol is as follows. Target DNA fragment, a rat insulin promoter (RIP) sequence (nucleotides -410 – 9), is amplified from rat genomic DNA by polymerase chain reaction (PCR). Recognition sites of restriction enzyme BglII and StuI are introduced into both terminus of the target DNA fragment by amplification using oligonucleotide primers. After cleaving with those two restriction enzymes, the DNA fragment is cloned into the upstream sequence of report gene luciferase (Luc) in plasmid pGL3-Luc (from Promega Corporation of USA). Thus, the report vector pGL3-RIP-Luc is constructed.

Report vector plasmid pGL3-RIP-Luc or control plasmid pGL3-Luc, together with plasmid pBabe-puro that expresses screening marker Puro, are cotransfected into RIN cells by calcium phosphate precipitation at a ratio of 10:1. By screening the transfected cells with Puromycin (5μg/ml), report cell line (RIN-RIP-Luc) containing report vector pGL3-RIP-Luc and control cell line (RIN-Luc) containing control pGL3-Luc are constructed.

To compare the biological activity of GLP-1, EX-4 or super-insulinotropic peptide-1 (SIP-1), RIN-Luc cells and RIN-RIP-Luc cells are cultured in serum-free medium for 24 hours, and then incubated with aforesaid peptides respectively at a final concentration of 10nM for 2 hours. The serum-free medium is then replaced by normal medium, and the cells are cultured for another 24 hours. The amount of luciferase activity in cell lysates is measured using fluorescence illuminometer (from Turner Designs Corporation of USA). The increased fold of luciferase biological activity, denoting the biological activity of the peptides, is calculated by dividing luciferase activity in cells with stimulation by luciferase activity in cells without stimulation. The greater the results, the higher activity the peptides have (results are shown in Fig.4).

Results show that, a certain level of luciferase expression can be detected in RIN-Luc control cells, but the level of luciferase activity does not change with peptide stimulation. However, in RIN-RIP-Luc cells, the luciferase activity is enhanced in response to peptide stimulation. These results suggest that the increase of luciferase activity by peptide stimulation is achieved through RIP promoter. It can also be seen from Fig.4 that SIP-1 has higher biological activity than EX-4 and GLP-1.

4) Stability assay for super-insulinotropic peptide-1.

To study the stability of super-insulinotropic peptide-1 *in vivo*, human blood plasma is used in this embodiment to mimic *in vivo* conditions. Add 2μg of the insulinotropic peptides each into 1ml of blood plasma and incubate at 37□ for 2 hours. Take a 50μl sample and add it into 1ml of the RIN-RIP-Luc cell medium (equivalent to a non-degraded concentration of 25nM). Percentage of preserved biological activity is calculated by dividing luciferase activity in treated cells by luciferase activity in untreated cells. Results are shown in Fig.5.

As demonstrated in Fig.5, GLP-1 has a very low stability and its activity is almost lost completely after incubation with human blood plasma at 37□ for 2 hours. However, the percentages of preserved biological activity of EX-4 and super-insulinotropic peptide-1 (SIP-1) are 77% and 78% respectively. These results suggest that the stability of super-insulinotropic peptide-1 is much higher than GLP-1 and similar to EX-4.

5) A drug compound containing super-insulinotropic peptide-1

To study the feasibility of using super-insulinotropic peptide-1 as a drug, in accordance with the normal method for preparing peptide drug compounds, a drug compound is prepared by adding manicol (5mg/ml) and human albumin (1mg/ml) into a solution containing super-insulinotropic peptide-1 (10μg/ml) and then adjusting the pH to 6-8 using phosphate buffer. After freezing and drying, the sample is more distinguishable and better looking, and its storage life is extended to 1 year or more without compromising its biological activity.

Preferred embodiment 2:

1) Amino acid sequence of super-insulinotropic peptide-2 (SIP-2):

SIP-2: NH_2-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

In this sequence, AA1 is Gly, AA2 is Glu, AA3 is Val, AA4 is Lys, and AA5 is Tyr.

2) Preparation of super-insulinotropic peptide-2 using solid-phase polypeptide synthesis method.

The protocol is identical to that for preferred embodiment 1.

To verify the correctness of the synthesised and purified product, the purified super-insulinotropic peptide-2 was subjected to mass chromatographic analysis, and the results are as follows:

Molecular formula: $C_{187}H_{281}N_{49}O_{60}S_1$; Theory molecular mass: 4207.68; Measured value: 4207.4.

These results confirm that the synthetic insulinotropic peptide-2 is the desired peptide.

3) Biological activity assay for super-insulinotropic peptide-2.

The protocol is identical to that for preferred embodiment 1. Results are shown in Fig.4.

As demonstrated in Fig.4, SIP-2 has higher biological activity than EX-4 and GLP-1.

4) Stability assay for super-insulinotropic peptide-2.

The protocol is identical to that for preferred embodiment 1. Assay results are shown in Fig.5.

As demonstrated in Fig.5, the stability of SIP-2 is much higher than GLP-1 and similar to EX-4.

Preferred embodiment 3:

1) Amino acid sequence of super-insulinotropic peptide-3 (SIP-3):

SIP-3: NH_2-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

In this sequence, AA1 is Ser, AA2 is Gln, AA3 is Val, AA4 is Lys, and AA5 is Tyr.

2) Preparation of super-insulinotropic peptide-3 using solid-phase polypeptide synthesis method.

The protocol is identical to that for preferred embodiment 1.

To verify the correctness of the synthesised and purified product, the purified super-insulinotropic peptide-3 was subjected to mass chromatographic analysis, and the results are as follows:

Molecular formula: $C_{188}H_{284}N_{50}O_{60}S_1$; Theory molecular mass: 4236.72; Measured value: 4236.3.

These results confirm that the synthetic insulinotropic peptide-3 is the desired peptide.

3) Biological activity assay for super-insulinotropic peptide-3.

The protocol is identical to that for preferred embodiment 1. Results are shown in Fig.4.

As demonstrated in Fig.4, SIP-3 has higher biological activity than EX-4 and GLP-1.

4) Stability assay for super-insulinotropic peptide-3.

The protocol is identical to that for preferred embodiment 1. Assay results are shown in Fig.5.

As demonstrated in Fig.5, the stability of SIP-3 is much higher than GLP-1 and similar to EX-4.

Preferred embodiment 4:

1) Amino acid sequence of super-insulinotropic peptide-4 (SIP-4):

SIP-4: Ac-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

In this sequence, AA1 is D-Ala, AA2 is Glu, AA3 is Val, AA4 is Lys, and AA5 is Tyr.

2) Preparation of super-insulinotropic peptide-4 using solid-phase polypeptide synthesis method.

The protocol is identical to that for preferred embodiment 1, except the acetylation of N-terminal using acetic anhydride when the synthesis is complete.

To verify the correctness of the synthesised and purified product, the purified super-insulinotropic peptide-4 was subjected to mass chromatographic analysis, and the results are as follows:

Molecular formula: $C_{190}H_{285}N_{49}O_{61}S_1$; Theory molecular mass: 4263.75; Measured value: 4263.9.

These results confirm that the synthetic insulinotropic peptide-4 is the desired peptide.

3) Biological activity assay for super-insulinotropic peptide-4 (SIP-4).

The protocol is identical to that for preferred embodiment 1. Results are shown in Fig.4.

As demonstrated in Fig.4, SIP-4 has higher biological activity than EX-4 and GLP-1.

4) Stability assay for super-insulinotropic peptide-4 (SIP-4).

The protocol is identical to that for preferred embodiment 1. Assay results are shown in Fig.5.

As demonstrated in Fig.5, the stability of SIP-4 is much higher than GLP-1 and notably higher than EX-4.

In summary, super-insulinotropic peptide-1-4 (SIP-1 – 4) introduced by this invention have higher insulinotropic activity and stability than prior art EX-4, and much higher stability than prior art GLP-1. Super-insulinotropic peptides introduced by this invention can therefore enhance drug efficacy while reducing required dosage thereby cutting toxic side effects, and can be used in potential clinical applications in the treatment of diabetes mellitus and obesity.

(Granted Claims)

Claims

1. A type of super-insulinotropic peptides, characterised in that: the amino acid sequence is NH2-His AA1 AA2 Gly Thr Phe Thr Ser Asp AA3 Ser AA4 AA5 Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-COOH, in which AA1 may be D-Ala, Ser, β-Ala or Gly; AA2 may be Glu, Gln or Asn; AA3 may be Val, Leu or Ile; AA4 may be Lys or Arg; and AA5 may be Tyr or Phe.
2. Super-insulinotropic peptides as described in Claim 1, characterised in that: aforesaid AA1 may be D-Ala or Gly; aforesaid AA2 may be Glu or Gln; aforesaid AA3 is Val; aforesaid AA4 is Lys; and aforesaid AA5 is Tyr.
3. Super-insulinotropic peptides as described in Claim 2, characterised in that: aforesaid AA1 is D-Ala; and aforesaid AA2 is Glu.
4. Super-insulinotropic peptides as described in Claim 1, characterised in that: the C-terminal may have amino group, benzyloxycarbonyl, dansyl or tertiary butyloxycarbonyl as protective group.
5. Super-insulinotropic peptides as described in Claim 4, characterised in that: the C-terminal has amino group as protective group.
6. Super-insulinotropic peptides as described in Claim 1, characterised in that: the N-terminal may have acetyl group, benzyloxycarbonyl, dansyl or tertiary butyloxycarbonyl as protective group.
7. Super-insulinotropic peptides as described in Claim 6, characterised in that: the N-terminal has acetyl group as protective group.
8. Super-insulinotropic peptides as described in Claim 1 – 7, characterised in that: the amino acid sequence is NH_2-His D-Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.
9. Super-insulinotropic peptides as described in Claims 1 – 7, characterised in that: the amino acid sequence is NH_2-His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

10. Super-insulinotropic peptides as described in Claims 1 – 7, characterised in that: the amino acid sequence is NH_2-His Ser Gln Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

11. Super-insulinotropic peptides as described in Claims 1 – 7, characterised in that: the amino acid sequence is Ac-His D-Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Lys Tyr Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser-$CONH_2$.

12. A drug compound, characterised in that: it contains one of the super-insulinotropic peptides as described in Claims 1 – 11 and other normal medicinal adjuvant.

13. A drug preparation, characterised in that: it contains one of the super-insulinotropic peptides as described in Claims 1 – 11 or drug compound as described in Claim 12; the form of medication may be injectable powder, injectable preparation, inhaler or oral application.

14. Any of the super-insulinotropic peptides as described in Claims 1 – 11 or drug compound as described in Claim 12 to be used as a drug preparation in the treatment of diabetes mellitus and obesity.